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Enzyme-mediated Crosslinking of Wool. Part I: Transglutaminase[1,2]

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Abstract Felting shrinkage of wool fabric can be controlled by oxidation and protease treatment, however, strength loss usually results. The Agricultural Research Service (ARS) process, providing bleaching, biopolishing, and shrinkage control by peroxycarboximidic acid oxidation and selective enzyme digestion of wool's scales can cause 10 to 18% strength loss. After ARS processing 3 to 5% fabric strength was regained with application of transglutaminase (TG). The TG reactivity involves the transferase-mediated, acyltransfer reaction between glutamine and lysine with the formation of carboxylamide groups of peptide-bound glutamine in wool keratin. Changes in the specular reflectance Fourier transform infrared spectra of the amide I and II regions attributed to the functional groups involved in the transamidation reaction provided evidence that cross-linking through TG had occurred in the fabrics. Optical and fluorescence microscopy showed no influence of TG on the anionic charge imparted by peroxycarboximidic acid oxidation and no influence of TG on the scale smoothing or removal of the enzymatic digestion involved in the ARS process. Confocal microscopy revealed the abundant presence of amine groups in the TG-treated fibers. Thus there is evidence that ARS-processed keratin substrates in the solid state can be self-cross-linked and that they have potential for further reactivity.

Key words ARS process, transglutaminase, specular reflectance FIER, scanning electron microscopy, enzyme-mediate crosslinking of wool

The ARS process, developed by the United States Department of Agriculture's Agricultural Research Service (ARS) for whitening, biopolishing, and shrinkproofing wool, is an effective and efficient two-step, chemoenzymatic process involving bleaching, biopolishing, and shrinkproofing. The pretreatment step of this process utilizes stabilized peroxycarboximidic acid, a powerful bleach when applied at pH 11.5 for 30 minutes at 30°C. A subse-

[1] The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

[2] U.S. Patent Application 20040261192 A1, December 30, 2004.

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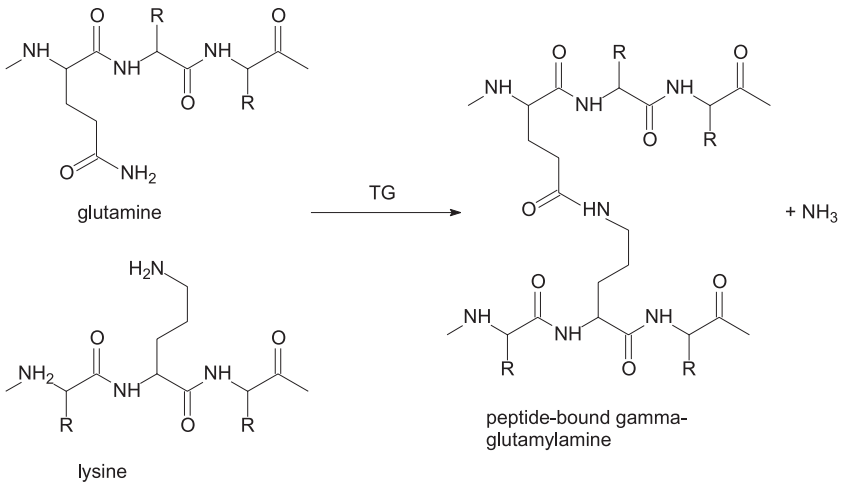


Figure 1 Transamidation reaction of enzyme-induced transglutaminase crosslinking: aminoacyltransferase catalysis with acyl transfer between peptide-bound glutamine (acyl-donor) and lysine primary amine (acyl acceptor) in the TG-mediated crosslinking of keratin in wool whereby isopeptide bonds form between γ-glutamyl carboxyl groups and the peptide-bound ε-amino groups of lysine residues to form ε(γ-glutamyl)lysine bonds.

quent treatment from a fresh bath utilizing either serine proteinase (Esperase™) with sodium sulfite or cysteine proteinase (raw papain or Liquipanol™, a papain enzyme formulation) without sodium sulfite, is effective for biopolishing and dimensionally stabilizing wool fabric to 3% or less area shrinkage. Scanning electron micrographs of the surface of ARS-treated wool fibers showed scale smoothing and removal with little effect on the cortical layer. The process provides a safe alternative to chlorination for shrinkage control and is effective on acetate, cotton, nylon, viscose and wool/cotton blended fabric. The isolated effects of the ARS formulation components in the pretreatment bath have been reported as were the effects of the full process on wool fiber, yarn, and fabric [1–4]. Reports from mill trials of strength losses of from 10 to 18% prompted us to investigate the effects of enzyme-mediated crosslinking as a third step to prevent strength loss. The crosslinking enzyme, transglutaminase (amine γ-glutamytransferase EC 2.3.2.13, referred to as TG), specifically, protein-glutamine gamma-glutamyltransferase, catalyzes an acyl transfer reaction between a donor substrate and an acceptor substrate. In the case of wool keratin, we reasoned that TG could catalyze an acyltransfer reaction between glutamine and lysine with the formation of carboxylamide groups of peptide-bound glutamine. In Figure 1, with the formation of intramolecular and intermolecular ε-(γ-Glu)-Lys crosslinks in keratin, fabric strength should be improved.

Commercial microbial TG typically is used to stabilize matrices in food systems such as bologna, sausage, and hot dogs to enhance hardness and elasticity and improve texture through crosslinking. The enzyme's active center is the sulfhydryl group (–SH). Thus the activity of TG is affected by oxidizing and reducing agents. TG catalysis can be utilized to convert soluble proteins to high molecular weight polymers and to improve protein functionality. For example, TG has been used on casein-finishing of leather [5]. It has been used to control the rheology of whey protein isolates [6]. This enzymatic protein can act as a biological glue, for example, to stabilize collagen in liver fibrosis [7], to maintain tissue integrity after cell injury, [8], and to reinforce the strength of collagen [9]. Active agents containing the amine functional group have been bound to glutamine in skin and hair [10]. Relevant work by Harding and Rogers established the role of TG-crosslinking in animal hair proteins [11]. When TG alone was applied to wool fiber before or after enzymatic treatment overall fiber damage was less when compared to treatment with enzyme alone or oxidative-enzyme treatment [12]. When TG was applied with enzyme, shrinkage was reduced by 44 to 47% to approximately 17% without losses in fabric weight or strength. Significant increase in the tensile strength of wool was found when TG was added as a pre- or post-treatment in enzymatic detergency [13]. A 25% increase in strength after applying TG to oxidized or reduced protease-treated wool yarn and fabric was evidence of crosslinking as documented by the incorporation into wool of the TG substrate, fluorescent primary amine fluoroscein cadaverine as visualized by confocal microscopy. [14, 15] When TG was included in enzymatic protease systems designed to control shrinkage the greater improvements in strength with TG treatments following the protease treatment was attributed to the formation of TG-mediated intra- and inter-isopeptide crosslinks (bonding of the ∈-amino group of a lysine residue to the γ-carboxyl group of a glutamic acid residue) [15]. There is reasonable expectation that by applying TG to ARS-oxidized and enzymatically treated wool, strength can be increased and that confocal microscopy with fluorescence of treated wool fibers can be used to document the extent of TG-mediated crosslinking.

Materials and Methods

Scoured and carbonized wool fibers, 21 micron, 1½″ domestic fleece was supplied by Bollman Hat Company, Adamstown, PA. Worsted wool woven fabric (Style #523,

twill weave), and wool jersey knit fabric (Style #532, jersey) were obtained from Testfabrics, Inc., West Pittston, PA. Four fabrics, each 10 g were cut to represent four replicates for each sample condition. All fabric samples were pretreated and treated in an Atlas LP2 Launder-Ometer and Lab Dyeing System in individual canisters containing bath volumes of 400 mL (liquor ratio, 10 : 1). The pretreatment step of the ARS process bleaches to a high level of whiteness and confers anionic charge to the wool after the application of 3 g/L NaOH, 1 g/L potassium gluconate (Sigma), 3 g/L dicyandiamide (Aldrich), 1 g/L Triton X-114 (7 to 8 ethylene oxide units, 1% solution cloud point, 22°C, Sigma), and 12 mL/L 50% hydrogen peroxide, at pH 11.5, for 30 minutes at 30°C. The subsequent treatment step involves selective enzymatic digestion of the scales of wool. The enzyme bath is constituted as 1.5% on weight of fiber (owf) Esperase 8.0 L™, a bacterial subtilisin serine protease, 548 TU/mg activity, (Novozymes North America, Inc. Franklinton, NC), Na_2SO_3 and triethanolamine buffer, pH 8–9, were applied at 45°C for 40 minutes. Alternatively, cysteine (thiol) protease, crude papain from Papaya Latex (Sigma) and/or commercial papain, 200 TU/mg activity Liquipanol T-200™ Enzyme Development Corporation, NY were applied at 2% owf in 20 mmol/L phosphate buffer, pH 7, with 1% owf Triton X-114 at 50°C for 60 minutes after the pretreatment step. After this process we applied Activa™ TG-TI, 38kD, microbial *Streptoverticillum mobaraense* isolate, (Ajinomoto Food Ingredients LLC, Ames, IA) from a fresh bath as 1.5% owf TG from a 1% in 50 mmol/L Tris(hydroxymethyl)aminomethane–HCl at pH 7 for 2 hours at 50°C with liquor ratio 10 : 1. TG is composed of 1% (w/w) enzyme containing 99% (w/w) maltodextrin with reported activity, 86–135 units/g, where one enzyme unit is defined as the amount causing the formation of 1 µmole of hydroxamic acid in 1 minute at 37°C. The optimal temperature for TG activity is 50–55°C and optimum pH stability is from pH 5 to pH 7.5 [16].

Property Measurements

All fabric samples were conditioned to constant relative humidity and temperature overnight before measurements were taken. Relaxation Shrinkage was measured for dimensional change after pretreatment/treatment according to ASTM D1284, "Relaxation and Consolidation Dimensional Changes of Stabilized Knit Wool Fabrics". The samples were soaked at room temperature in 0.1% Triton X-114 solution for 4 hours to relax them. They were dried and conditioned before measuring. Felting shrinkage was measured after five machine wash/five tumble-dry cycles using the AATCC Test Method 135-1992, Dimensional Changes in Automatic Home Laundering of Woven and Knit Fabrics. A Kenmore™ washing machine (Sears Company) with 1 cup of Woolite® fabric wash was operated in the delicate wash (permanent press setting) at 35°C for a 30-minute wash/rinse cycle, followed by machine dry (permanent press setting).

A bursting strength tester (SDL International P1000 M229B) was utilized for measuring dry and wet bursting strength, and four measurements were taken from each specimen, two samples were tested and a total of eight measurements were averaged. Whiteness index before and after treatment was measured using the color-insight QC Manager system (BYK-Gardner, Inc.) and ASTM E313 test method. For woven fabrics, tensile strength was tested on Instron Model 1122 Analyzer using $1'' \times 3''$ raveled strip with 1000 N load cell, five strips of woven fabric were taken from each specimen. A bursting strength tester (SDL International P1000 M229B) was utilized for measuring the bursting strength of the knitted fabric samples, four measurements were taken from each specimen.

Confocal Microscopy

Confocal optical microscopy was used to determine the anionic charge on the ARS-processed wool yarn cross-sections stained with 0.04 g Rhodamine B (Basic Violet 10, cationic dye), rinsed in tetrachloroethylene, and dried. The cross-sections were prepared on the Micro No. 200-A Microtome (Micro Instrument, Marshfield Hills, MA) using collodion embedding medium (Mallinckrodt, Paris, KY). Cross-sections of 5 µm thickness were visualized as confocal fluorescent images by a Leica TCS Confocal System, equipped with an HCX PL40X 1.25 NA lens using an excitation wavelength of 488 nm and an emission wavelength of 540–580 nm.

Confocal fluorescence microscopy was used to determine the effect of TG on wool. The fluorochromes, fluorescein cadaverine and fluorescein-5-EX, succinimidyl ester (Invitrogen, Molecular Probes, Inc., Eugene, OR), excitation 470–500 nm and emission 510–535 nm, were used to treat wool fibers for the detection of available amine groups needed for TG transcatalysis crosslinking of wool keratin. Stock solutions of each fluorochrome were prepared in sodium bicarbonate buffer (10 mg/mL) at pH 8.3. Each 2-mg wool sample was treated at room temperature with a 250 µL aliquot sample of fluorochrome for 5 hours. After rinsing individually in the buffer and rinsing several times with vortexing, the samples were extracted from clear solutions. After air drying they were mounted in Collodion™ (J.T. Baker, Phillipsburg, NJ) and cross-sectioned to 5 µm thickness on a Micro No. 200-A Microtome (Micro Instrument Division of KFK Machine, Marshfield, MA). Confocal images were collected from a Leica Microsystems Inc. microscope equipped with fluorescence capability, Model: TCS-SP Confocal Laser Scanning Microscope, manufactured by Leica Microsystems Inc. (Exton, PA) with excitation at 488 nm at 15% intensity and emission at 525 to 625 nm at 63× magnification in water mount using a 63× magnification water mount.

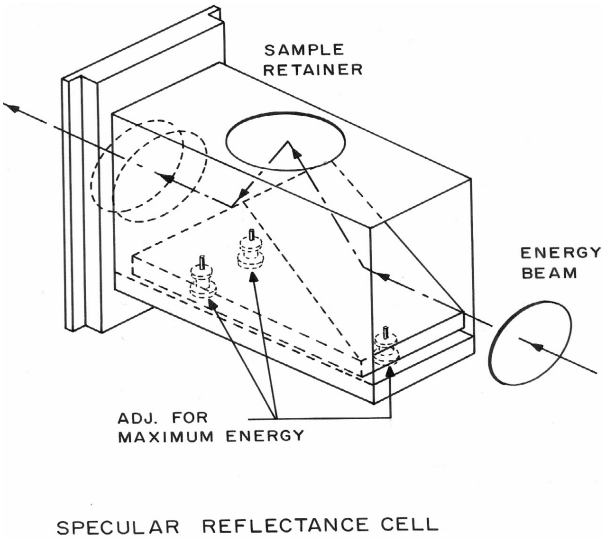


Figure 2 Reflection absorbance FTIR sample cell holder for fabric presentation [17].

Spectroscopy

Specular reflectance infrared spectra were collected on a Nicolet Magna System 560 spectrometer equipped with a specular reflectance sample cell holder, configured with two mirror planes that meet at an apex above which fabric samples, 1¾″ × 2″ are placed on top of the ½″ orifice above the apex in Figure 2. Note the IR energy beam impinges on one of the 30°-configured mirror planes and is reflected onto the sample. The sample is backed with a mirror to allow the beam to be reflected off the backing mirror and onto the second 30° mirror plane to be sent to the mercury cadmium telluride detector with transmission window, 4000 to 500 cm^{-1} whereby 256 scans for a total of 8192 fast Fourier transform (FFT) data points were collected, each at 4 cm^{-1} resolution. A mirror spectrum, collected for each sample, was used to ratio each single-beam spectrum.

Results and Discussion

Physical Properties

Blank samples (fabric in water medium without chemicals under reaction conditions) and control samples (as received) were included in the study. Relaxation shrinkage results from induced stress and strain imparted in yarn and/or fabric manufacture and in processing, especially finishing. Figure 3a and b shows the contribution relaxation shrinkage can make to overall shrinkage that includes felting shrinkage. It must be mentioned that the ARS process specifically controls felting shrinkage; that is, the dimensional stability of relaxed fabrics when they are subjected to laun-

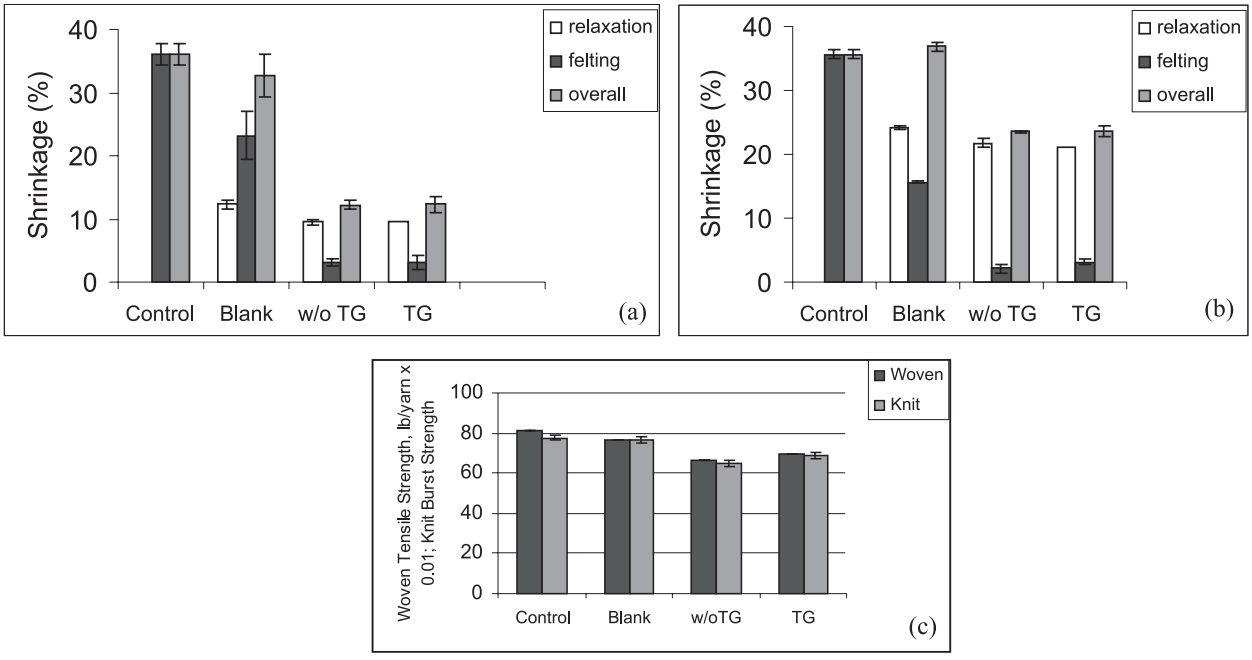
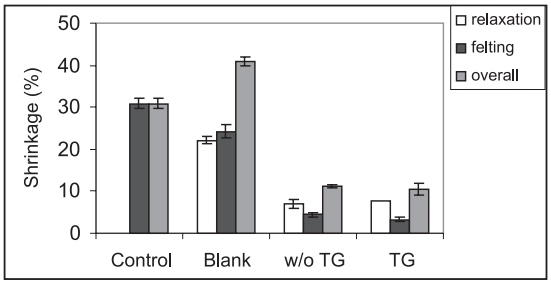
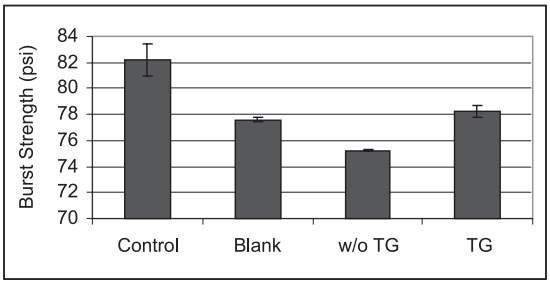


Figure 3 (a–c) Effects of TG on shrinkage and strength of wool fabrics with Esperase™ used in ARS-processing: (a) shrinkage of twill woven fabric; (b) shrinkage of jersey knit fabric; (c) strengths of woven and knit wool fabrics.



a.



b.

Figure 4 (a and b): ARS-Processed wool fabrics: effects of subsequent TG treatment: a – Liquipanol T-200™ papain enzyme: shrinkage of jersey knit fabric: b – strength of knit wool fabrics.

dering. TG applications to ARS wool processed wool fabrics showed improvement in fabric strength in Figure 3c.

Note the high contribution that relaxation shrinkage makes to the overall shrinkage, the higher relaxation shrinkage in the knit fabric relative to the woven, and the effectiveness of the ARS process to control shrinkage to 3% without the influence of TG. After applying TG to ARS processed knit and woven fabrics, approximately 4 to 5% strength was regained relative to the value of either blank or control fabrics.

Similarly, Liquipanol T-200™ papain was applied in the ARS process in place of Esperase™ enzyme in Figure 4a and b.

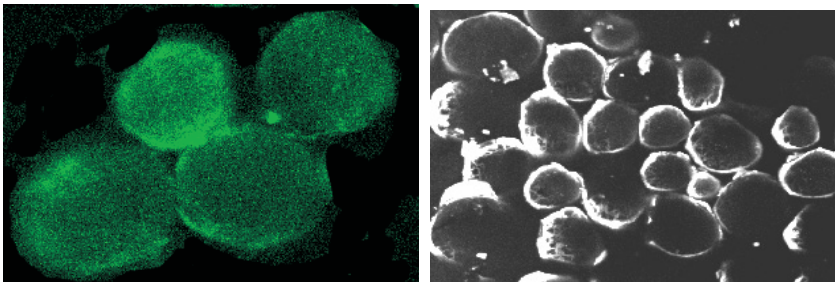
After applying TG to ARS processed knit fabrics treated with Liquipanol T-200™, no effect on shrinkage control was observed; however, approximately 3 to 4% strength was regained relative to the value of either blank or control fabrics.

Confocal Microscopy

The advantage of using confocal microscopy is that wool in its natural state can be observed as in-depth optical sections by longitudinal and transverse optical sectioning without the dehydration and coating required in scanning electron microscopy. Two channels, optical and fluorescence can be utilized. When fluorochromes are used to stain the specimen, the internal; structure can be revealed to indicate routes of penetration into and selective changes of fiber structure. Fluorescent Rhodamine B, cationic dye, was used to show diffuse anionic charge distribution throughout the untreated wool fiber due to sulfur's electron-pair distribution in Figure 5a. By contrast, after ARS oxidative bleaching, in Figure 5b, Rhodamine B was strongly and selectively absorbed by sulfoxylate groups of the cuticle to indicate localization of anionic charge on the fiber surface. Apparently the dye did not penetrate into the cortex.

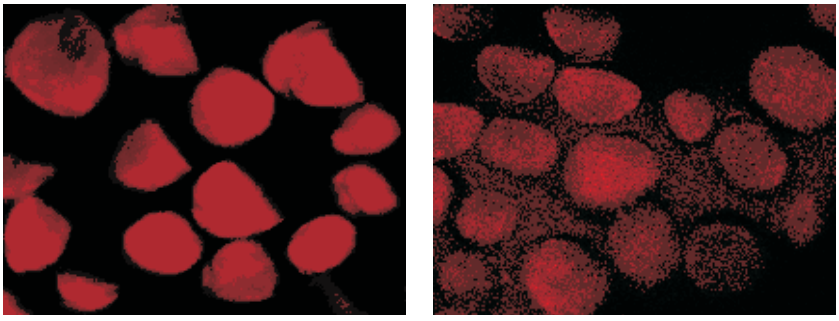
We compared fibers extracted from the yarns of untreated and ARS-treated fabric that had the subsequent TG treatment described above using fluorescein cadaverine and fluorescein-5-EX, succinimidyl ester fluorochromes. These fluorochromes, as amine-reactive reagents, can label non-protonated aliphatic amine groups, including terminal amines and the ε-amino group of lysines.

We used Fluorescein as an amine-reactive probe to react with lysine's ε-amino group. After exposure to TG we applied the fluorochrome to detect whether these amino groups were affected by TG-assisted inter- and/or intramolecular crosslinking. In Figure 6a there is bright, uniform fluorescence in untreated wool and in Figure 6b there is dull, nonuniform fluorescence to indicate that TG was effective in mediating transamidation trough amine functionality.



(a) (b)

Figure 5 (a and b): Confocal images from Rhodamine B-stained wool samples: (a) control, as received: (b) ARS-pretreated fiber after peroxycarboximidic acid oxidation through ARS processing and subsequent treatment with TG.



(a) (b)

Figure 6 (a and b): Confocal fluorescence images of wool fibers from woven twill fabric after staining with fluorescein-5-EX, succinimidyl ester fluorochrome: (a) Untreated wool fibers; (b) ARS-treated fibers subjected to TG treatment.

FTIR Spectroscopy

In Figure 7a–d infrared spectroscopy was used to probe protein molecular structure at the secondary level (the underlying structure of alpha-helical keratin consisting of a complex pattern of covalent bonds). Amide I (1700–1600 cm^{-1}), II (1500–1600 cm^{-1}), and III (1200–1320 cm^{-1}) absorbances were examined within the peptide CONH group. Amide III absorbances are relatively the strongest and they correspond to the C=O stretch weakly coupled with the C–N stretch and the N–H bending, whereas the amide II region represents C–N stretch strongly coupled with N–H bending, and the amide III region is N–H in-plane bending coupled with C–N stretching and also includes C–H and N–H deformation vibrations [18, 19]. Based on the pathway for TG transamidation shown in Figure 1 this approach is particularly applicable. Derivative spectroscopy provided qualitative information on discrete peak absorptions and curve-fitting provided quantitative analysis to make relative comparisons among wool knit fabrics: untreated (CK2), ARS-treated with Esperase 8.0 L™ (K2), and K2 followed by treatment with TG (K6). The aliphatic amide in glutamine should show the "amide I" strong C=O stretching at 1650–1690 cm^{-1}, amide II band, NH_2 bending at 1640–1600 cm^{-1} and the amide III band, C–N stretching at 1420–1405 cm^{-1}. The IR contributions for $–NH_2$ are weak absorbances within the range of 3550–3000 cm^{-1}. Other spectral regions of interest, 1120–1020 cm^{-1} represents sulfur-oxygen vibrations attributed to cystine oxidation in the bleaching step of the ARS process.

The second-derivative spectra were used to determine the number of bands and their positions in order to apply curve-fitting to resolve the individual bands that fit the spectral area of interest. This process involved iterating band height, position, and bandwidth at half-height to determine individual parameters in order to achieve the best Gaussian-shapes curve that fit the original spectra. The display of the family of bands comprising the overall band allows for analysis of the individual component bands.

In Figure 7e the resolved IR bands #1 to #12 were normalized to band #9 that showed no absorption change among the samples, CK2, K2, and K6. Note that in Figure 7e the absorbances of CK2 and K2 showed no change in the amide region after the application of the ARS process. However, by following the ARS process with TG treatment (K6 fabrics) there were increases in the amide bands #2, #3 and #4 (amide I and II).

In Figure 7f, the ARS treated fabric, K2, showed the development of sulfoxide bands, as expected from the oxidation of wool, that is, formation of $–SO_2–S–$ (1118 cm^{-1}), $–SO_3–$ from cysteic acid (1040 cm^{-1}), and $–S–SO_3–$ (1024 cm^{-1}) [20, 21].

Conclusions

The transamidating activity of TG was observed in the self-crosslinking of wool. TG was effective for mediating in-situ crosslinking of ARS-processed wool fabric when applied from fresh baths at 50°C for 2 hours at pH 7. Evidence of the reaction of transamidation, the crosslinking wool's amino acids, glutamine and lysine, to form ε(γ-glutamyl)lysine bonds, was apparent from images from fluorescence confocal microscopy. Yet in terms of potential reactivity, after TG-mediated crosslinking, the persistence of fluorescence indicated the presence of available amine groups. Confocal microscopy utilizing cationic fluorescent dye, Rhodamine B, showed that the anionic surface charge of ARS-processed fibers had been preserved after treatment with TG with no impediment to further reactivity. Scanning electron microscopy images (not shown) indicated no apparent structural changes. The TG-treated fabrics remained biopolished with no alteration in scale smoothing or removal and the structural integrity of the fibers remained intact. A moderate increase of 3 to 5% in fabric strength was consistent with TG-mediated crosslinking of wool.

Examination of the amide I and II regions of the infrared spectra provided substantive evidence that cross-linking through TG had occurred in the fabrics. In the expanded IR regions of the spectra (1750–1350 cm^{-1}) there were discrete changes in the amide I and II bands when control (CK2), ARS-processed (K2), and ARS-processed and TG-treated (K6) samples were compared. Specifically, the band differences in the amide I region, 1750–1650 cm^{-1},

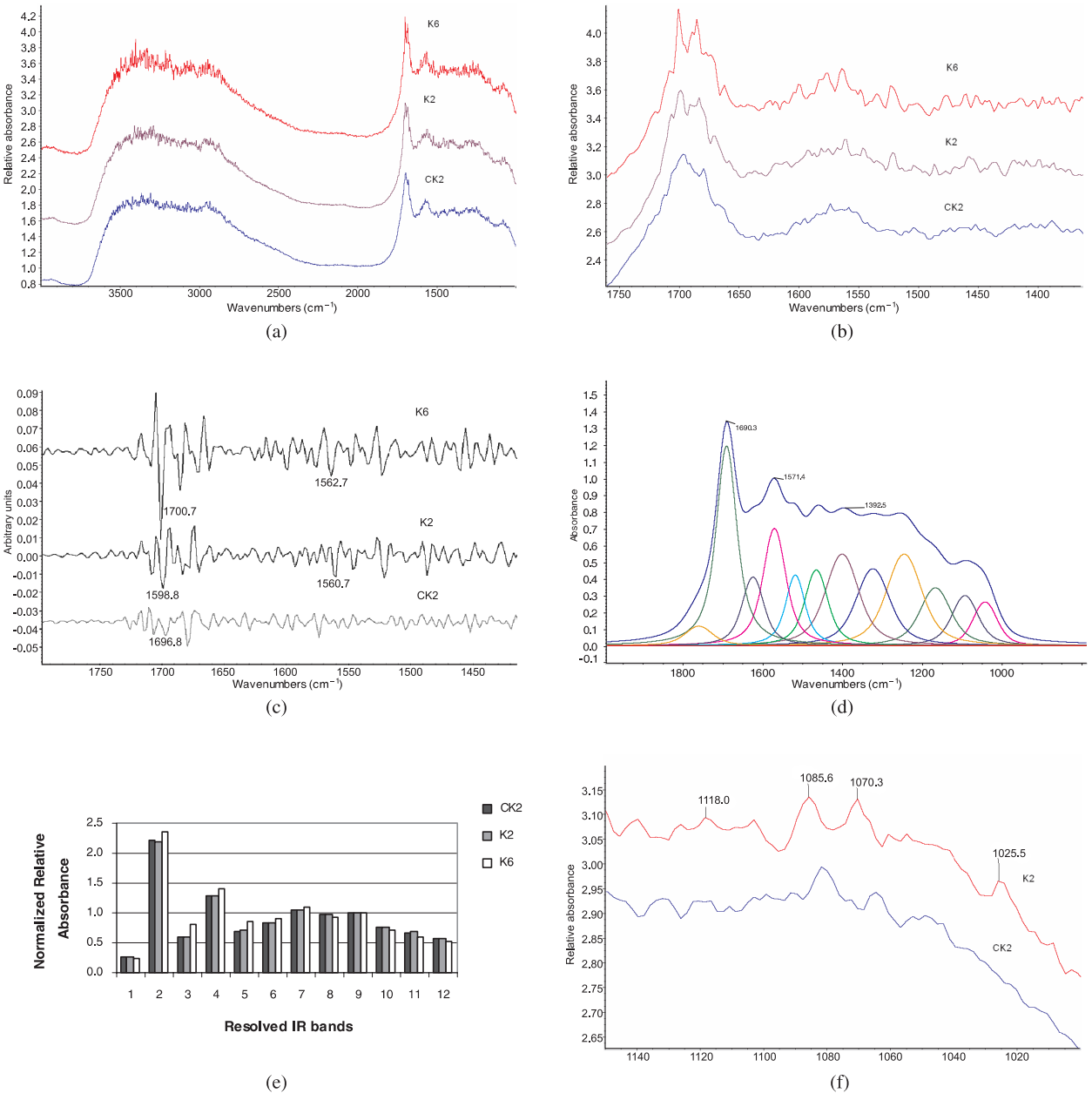


Figure 7 (a–f): FTIR reflection absorbance spectra of untreated (CK2), ARS-treated (K2), and ARS-treated followed by TG treatment (K6) wool knit fabrics: (a) full absorption range showing NH_2 bands (3550–3000 cm^{-1}), amide bands (1700–1500 cm^{-1}), and sulfoxide bands (1120–1040 cm^{-1}); (b) expanded Amide I, II, and III absorption regions; (c) second derivative spectra expanded in the amide region; (d) curve-fit, resolution of IR bands for sample K6; (e) relative absorptions for CK2, K2, and K6 from curve-fit data in (c); (f) sulfoxide-expanded absorption regions for CK2 and K2.

in Figure 7b pointed the way to further examination by second-derivative spectral analysis for isolating the distinctive bands of interest in Figure 7c. The amide I band signaled changes within the CONH group of glutamine, the site of proposed TG cross-linking mediation. After peak resolution through curve-fitting in Figure 7d of the K6 spectrum and normalization with the 1250 cm^{-1} band as reference, it was apparent that significant changes had occurred in the

salient amide I and amide II absorbances. These changes were then attributed to the functional groups involved in the proposed transamidation reaction of enzyme-mediated TG crosslinking of wool.

Evidence is therefore provided that keratin substrates in the solid state can be self-cross-linked and that there is the potential for the self-cross-linking of other keratinaceous materials.

Acknowledgements

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